

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C07K 16/08, 16/18, 16/28, 16/30, 16/24, A61K 39/395	A2	(11) International Publication Number: WO 00/46251 (43) International Publication Date: 10 August 2000 (10.08.00)
(21) International Application Number: PCT/EP00/00933 (22) International Filing Date: 4 February 2000 (04.02.00) (30) Priority Data: 60/118,810 5 February 1999 (05.02.99) US 60/131,398 28 April 1999 (28.04.99) US 60/134,674 18 May 1999 (18.05.99) US (71)(72) Applicant and Inventor: BÜLOW, Jens-Ulrich [DE/DE]; Flughafenstrasse 19, D-76140 Karlsruhe (DE). (74) Agent: OTTEVANGERS, S.U.; Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: HUMAN POLYCLONAL ANTIBODIES FROM GENETICALLY ENGINEERED ANIMALS (57) Abstract Substantially human antisera are provided by genetically modifying a domestic animal generally weighing at least about 1 kg. The domestic animal is genetically modified by generating inactive heavy and light chain immunoglobulin loci and integrating at least functional portions of the human heavy and light chain immunoglobulin loci, whereby the human loci generate an immune response. The antisera find use in the treatment of diseases, immunocompromised patients and in case of transplantation.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Title: HUMAN POLYCLONAL ANTIBODIES FROM GENETICALLY
ENGINEERED ANIMALS

INTRODUCTION

Field of the Invention

5 The field of this invention is substantially human polyclonal antisera for prophylactic and therapeutic treatment of humans.

Background

10

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. At the same
15 time the control of infections is threatened by the emergence of new pathogens. The increasing number of immunocompromised individuals due to malnutrition, AIDS, medical therapies of cancer, autoimmune diseases and organ transplantation decreases the efficacy of antibiotic therapy and increases
20 the difficulty of controlling infections.

Therapies of patients with malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high.

25 Advances in monoclonal antibody technology have provided little improvement because of the immunogenicity of the monoclonal antibodies and their lack of potency. Anti-idiotypic antibody responses in patients undergoing monoclonal antibody therapy can render the antibody therapy
30 ineffective.

Therapy of steroid resistant rejection of transplanted organs requires the uses of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing

alloimmune response in the transplant recipient. However, immunogenicity of antibody preparations may render such therapy ineffective and prevent rejection reversal. As a consequence, a transplanted organ may be rejected.

5 Similarly, antibody therapies of autoimmune disease patients are of limited success due to the immunogenicity of antibody preparations. While humanization of antibodies decreases immunogenicity, the effectiveness of such antibodies is limited by anti-idiotypic antibody responses and the lack of
10 potency of monoclonal antibodies. Non-immunogenic, potent reagents for the modulation of immune responses have to be developed.

Polyclonal antibody therapy for the treatment of infectious
15 diseases was introduced at the end of the last century. By the 1930s, serum therapy was used for treatment of bacterial and viral infections including pneumonia, meningitis, scarlet fever, whooping cough, anthrax, botulism, gangrene, tetanus, brucellosis, dysentery, tularemia, diphtheria, measles,
20 poliomyelitis mumps and chickenpox. However, the systemic administration of animal sera caused fevers, chills, and allergic reactions. Serum sickness occurred in 10-50% of treated individuals.

25 The potential of using antibodies in the treatment of a variety of indications is very high. The ability to specifically bind to a target entity provides diverse opportunities to sequester and destroy the entity. However, as demonstrated above, there have been many impediments to
30 the use of heterologous and humanized antibodies. The limitations of monoclonal antibodies adds the additional impediment of reduced affinity. Thus, there is a pressing need to find alternative modalities which provide protection against infectious disease and malignancies or the
35 immunomodulation of transplant recipients and autoimmune disease patients.

Relevant Literature

Antibody-based therapies in infectious diseases were recently
5 reviewed by A. Casadevall and M. D. Scharff, Clinical
Infectious Diseases 1995; 150-161.

The use of antibodies for the treatment of cancer and
malignancies was recently reviewed by C. Botti, A. Marinetti,
10 S. Nerini-Molteni, and L. Ferrari, Int J Biol Markers 1997;
12(4):141-147; D.R. Anderson, A. Grillo-Lopez, C. Varns, and
K.S. Chambers, Biochem Soc Trans 1997; 25(2):705-708; C.
Renner, L. Trumper, and M. Pfreundschuh, Leukemia 1997; 11
Suppl 2:S55-59; B. Bodey, S.E. Siegel, and H.E. Kaiser,
15 Anticancer Res 1996; 16(2):661-674.

The use of polyclonal antibody preparations for the treatment
of transplant rejection was recently reviewed by N. Bonnefoy-
Berard and J.P. Revillard, J Heart Lung Transplant 1996;
20 15(5):435-442; C. Colby, C.A. Stoukides, and T.R. Spitzer,
Ann Pharmacother 1996; 30(10):1164-1174; M.J. Dugan, T.E.
DeFor, M. Steinbuch, and A.H. Filipovich, Ann Hematol 1997;
75(1-2):41-46.

25 The use of polyclonal antibody therapies for autoimmune
diseases has been described by W. Cendrowski, Boll Ist
Sieroter Milan 1997; 58(4):339-343; L.K. Kastrukoff, D.R.
McLean, and T.A. McPherson, Can J Neurol Sci 1978; 5(2):175-
178; J.E. Walker, M.M. Hoehn, and N. Kashiwagi, J Neurol Sci
30 1976; 29(2-4):303-309.

The depletion of fat cells using antibody preparations has
been described by L. De Clercq, J. Mourot, C. Genart, V.
Davidts, and C. Boone, J Anim Sci 1997; 75(7):1791-1797; J.T.
35 Wright and G.J. Hausman, Obes Res 1995; 3(3):265-272.

The cloning of animals from cells has been described by T. Wakayama, A.C.F. Perry, M. Zuccotti, K.R. Johnson and R. Yanagachi, *Nature* 1998; 394:369-374; J.B. Cibelli, S.L. Stice, P.J. Golueke, J.J. Kane, J. Jerry, C. Blackwell, A. Ponce de Leon, and J.M. Robl, *Science* 1998; 280:1256-1258; J.B. Cibelli, S. L. Stice, P. J. Golueke, J.K. Kane, J. Jerry, C. Blackwell, F. Abel de Leon, and J. Robl, *Nature Biotechnology* 1998; 16:642-646; A. E. Schnieke, A.J. Kind, W.A. Ritchie, K. Mycock, A.R. Scott, M. Ritchie, I. Wilmut, A. Colman A, and K.H. Campbell, *Science* 1997; 278(5346):2130-2133; K.H. Campbell, J. McWhir, W.A. Ritchie, and I. Wilmut, *Nature* 1996; 380(6569):64-66.

Production of antibodies from transgenic animals is described in U.S. Patent nos. 5,814,318; 5,545,807; and 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent no. 5,416,260. A method for introducing DNA into an embryo is described in U.S. Patent no. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent no. 5,453,357.

SUMMARY OF THE INVENTION

Methods are provided for the production of substantially human polyclonal antisera to a specific antigen, where a transgenic domestic animal comprising genetically altered light and heavy chain immunoglobulin loci and at least a portion of human light and heavy chain immunoglobulin loci are provided. The method employs stepwise modification of a domestic animal in which the antibody repertoire is diversified predominantly by gene conversion (e.g rabbits, sheep, pigs, cows). The method involves replacement by homologous recombination of endogenous elements of the immunoglobulin loci with the corresponding human counterparts, in particular, replacement of one or several exons encoding constant regions of heavy and light chain and

one or several variable region elements including the one proximal to the D region locus. In animals, where antibody diversity is generated predominantly by gene conversion, replacement of the V region most proximal to the D region with a human V region element results in expression of the human V element in the majority of immunoglobulins. This genetic engineering is followed by breeding hosts of the same species and selecting for a host which is capable of responding to immunization with production of substantially human antisera with host glycosylation, the immunoglobulin having at least a functional portion of the human heavy chain. Animals expressing the substantially human protein sequence immunoglobulins are used for the generation of polyclonal antibody preparations by immunization with immunogens of interest, particularly, immunogens which initiate antibody production which has therapeutic activity. After purification of the antisera, such antisera may be used, by itself or in combination, with other reagents for the depletion of infectious reagents, malignant cells, cancers, undesirable target cells or immunomodulation.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for producing substantially human antisera in a heterologous host by immunizing the host with an immunogen. The host is characterized by; being at least substantially incapable of producing endogenous antisera and capable of predominantly producing substantially human polypeptide antisera upon exposure to an immunogenic substance; and retaining its capability of rearranging the immunoglobulin locus and recombining the V, (D_H), J and C regions to produce substantially human protein antisera, which include at least one human immunoglobulin constant region and/or at least one human variable (V) region element. Of particular interest are constant regions of the subclasses of C_α or C_γ, including any of the C_γ subclasses 1, 2, 3 and 4.

DNA fragments encoding human constant regions and variable elements are integrated into the genome by homologous recombination and replace the corresponding endogenous elements.

5

Various animals, particularly domestic animals, which can provide reasonable volumes of antisera may be employed. The animals generally are at least 1 kg, preferably 2 kg, and may be 5 kg or more when adult, although smaller animals can be used as appropriate. Also the gestation period should be less than 12 months, usually being in the range of 1 to 4 months. Illustrative animals include *Lagomorpha*, e.g. rabbit, ovine, bovine, canine, feline, equine, and the like, excluding murine. Of particular interest are animals where diversification of the antibody repertoire is accomplished predominantly by gene conversion (i.e. rabbits, pigs, sheep, cattle). In these animals, replacement of the V region element proximal to the D region with a human V region element will result in the expression of the human V region element in the majority of immunoglobulins. The described genetic engineering approach of the subject invention is substantially easier than other approaches that have been performed with mice. In mice, however, diversification of the antibody repertoire is accomplished predominantly by gene rearrangement.

Host cells, e.g. fibroblasts, keratinocytes, myocytes, hepatocytes, epithelial cells, or other cells which may be grown and expanded in culture and do not have a rearranged genome, are transformed (genetically modified) by the introduction of DNA fragments into the cells, where the fragments become integrated into the host genome. Introduction may be by a variety of methods, including bare DNA, transfection with a viral vector, fusion, biolistics, liposomes, etc. The particular method will be selected in accordance with the purpose of the introduction of the DNA

30

35

and the efficiency of integration. Functional immunoglobulin light and heavy chain loci are modified by homologous recombination, by replacing at least a portion of the host heavy chain constant region with at least a functional
5 portion of the human heavy chain constant region and if desired, analogously, the host light chain constant region with a human light chain constant region. Of particular interest is also the replacement of the V region most proximal to the D region with a human V region element. In
10 this way, while some portions of the immunoglobulin are host sequences, the antisera is not likely to cause a strong immune response in view of the great variety of variable regions in the antisera. In animals, where antibody diversity is generated predominantly by gene conversion,
15 replacement of the V region most proximal to the D region with a human V region element results in expression of the human V element in the majority of immunoglobulins. For the replacement of constant regions it is of particular interest to include at least about 2 of the 3 domains C_{H1} , C_{H2} , and C_{H3} ,
20 of the constant region, particularly including C_{H3} . To the extent such antisera can function in a host, particularly an immunocompromised host, the reduced number of stages to attain hosts which produce such antisera is attractive.

25 For integration at a predetermined site, constructs are prepared which include, in sequence, the DNA fragment for integration and a first marker gene bordered by homologous sequences of at least about 30nt and a second marker gene, whereby homologous integration results in loss of the second
30 marker gene. By having the second marker gene providing negative selection--cells with the second marker gene are selected against and removed from the cell mixture; by having the first marker gene providing positive selection--cells having the first marker gene are retained--by using a medium
35 to which the second marker gene is sensitive. In this manner, those cells in which the construct is randomly

integrated are decreased. By following with a medium selective for the first marker gene, cells not retaining the first marker gene will be decreased. In this way, the remaining cells should be those having homologous recombination. Desirably, the cells are in a rapidly proliferating status, rather than a non-proliferating status. By employing a growth medium, such as RPMI1640 or DMEM, supplemented with FCS and growth factors, a growth cycle can be induced.

After the cells have been transformed or transfected, the cells are put in a selective medium in accordance with the marker employed, usually an antibiotic resistance or the tk gene. The cells are expanded in culture and then cloned. Individual cells in clones may then be screened for the desired genetic modification. Conveniently, PCR may be used to identify that the desired modification, deletion or integration, has taken place.

The genetic modifications may be a single modification or, if desired, after expansion of cells having the first modification, the cells may then be subjected to a second modification. For example, after replacing the heavy chain constant regions, one could replace the light chain constant regions.

Where an individual modification occurs, one can use a single marker for positive selection and use the same marker repetitively. Where two or more modifications to the same cell are generated, different positive selection markers should be used, in order to independently select at each stage. As already indicated, there are numerous antibiotic resistance genes, which genes may be used in combination, allowing for selection at each stage. Genes useful for selection include neo, tet, cam, tk, pen, mtX, etc. After the host cells have been modified and demonstrated to have

the desired modification, the cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Alternatively, enucleation of oocytes and nuclear transfer can be performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369) The resulting functional egg cells are then cultivated in an appropriate medium and transferred into synchronized recipients.

Another method for producing nuclear transfer unit cells is to introduce DNA constructs comprising human transgenes into fertilized eggs. The eggs may then be expanded to provide embryonic stem cells, which are screened for the desired genetic modification and subsequent embryo transfer into foster mothers, where the eggs are brought to term, and the resulting neonates screened for the modified genotype.

The resulting mutated hosts may then be used for breeding with other mutated hosts. For example, hosts having an altered heavy chain immunoglobulin locus may be bred with hosts having an altered light chain immunoglobulin locus to breed a host capable of producing substantially human polypeptide immunoglobulins. The hemizygous siblings containing the two mutated genes are then bred to produce homozygous siblings. Homozygosity may be readily determined by the absence of the undesired gene sequences. After each breeding, the host is assayed for the presence of the genetic modification in its cells, particularly the germ cells, and may be bred to a further generation, usually not more than three generations, to ensure that the modification is stably maintained through successive generations. The genomes of the various offspring may be analyzed for the maintenance of the genetic modifications or, as appropriate, the offspring

may be analyzed for the biological change which the genetic modification generated.

Once the host has been generated, the host may now be
5 used to produce antisera under a variety of conditions.
Depending upon the use of the antisera, antigens, immunogens
comprising a hapten covalently bonded to an antigen,
organisms, e.g. viruses and unicellular organisms, alive,
attenuated or dead, fragments of organisms, organelles,
10 cells, particularly human cells or fragments of cells, or the
like may be used. Thus the antisera may be directed to an
antigen, a small organic molecule or a cell, where the
various entities may be endogenous or exogenous to the human
host. The immunization composition may be administered in
15 any convenient manner, with or without an adjuvant, and may
be administered in accordance with a predetermined schedule.
The affinity for the immunization composition may then be
monitored and the antisera collected when the antisera has
the desired specificity and affinity. The affinity of the
20 antisera generally will be at least about 10^{-7} , usually at
least about 10^{-8} , preferably at least about 10^{-9} , or higher.

For some application, one may use hosts in which the V
element proximal to the D regions has been replaced with
25 various human V region elements. In this way, different
immune responses to the same immunogen will be obtained from
the different hosts, where the variable region sequence may
be as a result of gene conversion, providing different
alleles. The antisera from the different hosts may be mixed
30 to provide a broader repertoire of antibodies. Up to 10 or
more different hosts may be employed, depending on the
antigen of interest.

Antibody preparations are obtained by fractionating blood of
35 genetically engineered animals expressing human sequence
immunoglobulins. A concentrated immunoglobulin fraction may

be prepared by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

5

The fractionated antibodies may be dissolved or diluted in non-toxic, non-pyrogenic media suitable for intravenous administration in humans, for instance, sterile buffered saline. In some applications, antibody preparations may be applied directly onto epithelium. For such applications, fractionated antibodies may be dissolved in a water soluble gel such as KY-jelly and the like. |

The antibody preparations used for administration are generally characterized by containing a polyclonal antibody population, having immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody preparation may contain immunoglobulins of various isotypes. Alternatively, the antibody preparation may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody preparation will consist of unmodified immunoglobulins. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody preparations generally are administered into the vascular system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody preparation is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be

accepted by the patient. Administration of the effective dosage may occur in a single infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody preparations are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

10

The antibody preparations find use in their ability to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably also are capable of stimulating an immune response.

20

Administration of an antibody preparation against an infectious agent as monotherapy or in combination with chemotherapy results in elimination of infectious particles. A single administration of antibodies decreases the number of infectious particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly, antibody therapy in patients with malignant disease as monotherapy or in combination with chemotherapy reduces the number of malignant cells generally 10 to 100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of time to assure the complete elimination of infectious particles, malignant cells, etc. In some instances, therapy with antibody preparations will be continued for extended amounts of time in the absence of detectable amounts of infectious particles or undesirable cells. Similarly, the use of antibody therapy for the modulation of immune responses may consist of single

35

or multiple administrations of therapeutic antibodies. Therapy may be continued for extended amounts of time in the absence of any disease symptoms.

- 5 The subject treatment may be employed in conjunction with chemotherapy at dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease patients or transplant recipients antibody therapy may be employed in conjunction with immunosuppressive therapy at dosages
10 sufficient to inhibit immune reactions.

The following examples are offered by way of illustration and not by way of limitation.

15 EXPERIMENTAL

Generation of transgenic rabbits expressing substantially human immunoglobulin

- Exons encoding human constant region elements and variable
20 region elements are integrated into the genome of rabbit fibroblasts by homologous recombination. Rabbit fibroblasts are transfected with various linearized DNA constructs containing human immunoglobulin locus elements. Successfully transfected cells are selected and used for the cloning of
25 rabbits.

Cloning of rabbits

- Mature Dutch Belton rabbits are superovulated by subcutaneous injection of follicle stimulating hormone (FSH) every 12
30 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by ovidual flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation.
35 Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St. Louis, MO) dye under ultraviolet light.

Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15 μ s (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6 μ M) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty Media, Lavalent, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 7 days at 37.8°C and 5%CO₂ in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

Binding of human antibodies expressed in rabbits to Hepatitis B surface antigen

Genetically engineered rabbits (as described above) are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (10 μ g in incomplete Freund's adjuvant) on day 0 and day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μ g/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μ l/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horse-radish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control serum from non-immunized rabbits is used. Serum from non-immunized rabbits does not react with HBsAg. At a

dilution of 1:100 the optical density measured in uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized rabbits contains substantially human antibodies reactive with HBsAg. At a serum dilution of 1:100 the
5 measured optical density is 2.8. Upon further dilution of the serum the measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce
10 substantially human anti-HBsAg antibodies following immunization.

Complement mediated cytotoxicity of virus infection cell line using human antibodies
15 A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi ^{51}Cr in 100 μl PBS for 1 hr at 37°C. Two thousand ^{51}Cr -labeled cells are incubated with serum from genetically engineered rabbits expressing anti-HBsAg immunoglobulin (see above). After two hours at 37°C the
20 release of ^{51}Cr into the supernatant is determined by measuring radioactivity using a scintillation counter. For the determination of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as follows:
$$\% \text{Lysis} = \frac{\text{CPM experimental} - \text{CPM\#spontaneous}}{\text{CPM\# total} - \text{CPM spontaneous}}$$

25 spontaneous. Incubation of labeled cells with serum (diluted 1:30) from non-immunized rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from immunized rabbits causes 80% cell lysis. Inactivation of
30 complement in the serum by heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive. These results demonstrate that substantially human antibodies produced by genetically engineered rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

Treatment of animal with infection.

Substantially human immunoglobulin is purified from the serum of genetically engineered rabbits by ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are
5 injected with one million human liver carcinoma cells expressing HBsAg. Subsequently, 25 µg immunoglobulin is injected peritoneally once per day. Animals treated with antibodies isolated from non-immunized rabbit serum die after about 60 days. This is similar to untreated recipients of
10 liver carcinoma cells. In contrast, mice treated with antibodies isolated from immunized rabbit serum survive for more than 150 days. This demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

15 It is evident from the above results that by using genetically engineered rabbits expressing substantially human immunoglobulin genes, polyclonal antibody preparations against antigens, infectious particles, cancer cells, and the
20 like can be generated. Such polyclonal antibody preparations can be used to treat patients suffering from an infectious disease or a malignancy. The antisera also can be used to modulate an immune response by elimination of cell sub-populations, cytokines, or the like. The human antibody
25 preparation has a substantially reduced likelihood of engendering an immune response in human patients, as compared to heterologous antisera, it will have few side effects and it can be used safely with positive results.

30 All of the references cited herein are incorporated herein by reference as if each reference was individually wholly incorporated.

It will be apparent to one of ordinary skill in the art that
35 many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A polyclonal antisera composition of a nonhuman animal that specifically recognizes an immunogen, wherein said antisera composition is comprised predominantly of substantially human immunoglobulin protein molecules
5 comprised of at least a portion of a human heavy chain polypeptide, wherein said substantially human immunoglobulin protein molecules specifically bind to said immunogen.
2. The polyclonal antisera according to Claim 1, wherein
10 said transgenic nonhuman animal is immunized with said antigenic entity, weighs at least 1 kg and comprises at least a portion of functional human heavy chain immunoglobulin genes integrated by homologous recombination into its genome.
- 15 3. The polyclonal antisera composition according to Claim 1, wherein said transgenic nonhuman animal generates antibody diversity predominately by gene conversion.
4. The polyclonal antisera composition according to Claim
20 1, wherein said transgenic nonhuman animal is from the order *Lagomorpha*.
5. The polyclonal antisera composition according to Claim 1, wherein said portion of functional human heavy chain
25 immunoglobulin genes comprises at least one constant region element.
6. The polyclonal antisera composition according to Claim 5, wherein said portion of functional human heavy chain
30 immunoglobulin genes further comprises at least one variable region element.

7. The polyclonal antisera composition according to Claim 6, wherein said variable region element is the variable region element proximal to the D region.

5 8. The polyclonal antisera composition according to Claim 1, wherein said immunogen comprises a disease causing organism or antigenic portion thereof.

9. The polyclonal antisera composition according to Claim
10 1, wherein said immunogen is an antigen endogenous to humans.

10. The polyclonal antisera composition according to Claim 1, wherein said immunogen is an antigen exogenous to humans.

15 11. A transgenic nonhuman animal weighing at least 1 kg and comprising at least a portion of functional human heavy chain immunoglobulin genes integrated by homologous recombination into its genome, wherein said portion of functional human heavy chain immunoglobulin genes rearranges in frame with
20 heavy chain immunoglobulin sequences endogenous to said nonhuman animal to encode functional, substantially human antibody molecules that comprise at least in part human heavy chain immunoglobulin polypeptide sequences, and wherein said animal predominantly produces said functional, substantially
25 human antibody molecules when immunized.

12 A transgenic nonhuman animal weighing at least 1 kg and comprising at least a portion of functional human light chain immunoglobulin genes integrated by homologous recombination
30 into its genome, wherein said human light chain immunoglobulin genes rearrange in frame with sequences endogenous to said nonhuman animal to encode functional, substantially human antibody molecules that comprise at least in part human light chain immunoglobulin polypeptide
35 sequences.

13. The transgenic nonhuman animal according to Claim 11 or 12, wherein said transgenic nonhuman animal generates antibody diversity predominately by gene conversion.
- 5 14. The transgenic nonhuman animal according to Claim 11 or 12, wherein said transgenic nonhuman animal is from the order *Lagomorpha*.
- 10 15. The transgenic nonhuman animal according to Claim 11 or 12, wherein said portion of functional human heavy chain immunoglobulin genes comprises at least one constant region element.
- 15 16. The transgenic nonhuman animal according to Claim 15, wherein said portion of functional human heavy chain immunoglobulin genes further comprises at least one variable region element.
- 20 17. The transgenic nonhuman animal according to Claim 16, wherein said variable region element is the variable region element proximal to the D region.
- 25 18. The transgenic nonhuman animal according to Claim 12, wherein said human immunoglobulin light chain gene encodes the κ chain.
19. An antisera composition produced by the transgenic nonhuman animal according to Claim 11.
- 30 20. A method for neutralizing an antigenic entity in a human body component, said method comprising:
contacting said body component with an antisera composition according to Claim 1, whereby said substantially human immunoglobulin protein molecules in said antisera composition
35 specifically bind and neutralize said antigenic entity.

21. The method according to Claim 20, wherein said antigenic entity is from an organism that causes an infectious disease.

22. The method according to Claim 20, wherein said antigenic
5 entity is a cell surface molecule.

23. The method according to Claim 22, wherein said cell surface molecule is from a lymphocyte or an adipocyte.

10 24. The method according to Claim 20, wherein said antigenic entity is a human cytokine or a human chemokine.

25. The method according to Claim 20, wherein said antigenic
entity is a cell surface molecule on a malignant cancer cell.

15

26. A method of producing a transgenic nonhuman animal weighing at least 1 kg and comprising human immunoglobulin genes integrated by homologous recombination into its genome, wherein said animal predominantly produces functional,
20 substantially human antibody molecules comprised at least in part of human immunoglobulin polypeptide sequences when immunized, said method comprising:

producing a first mutated animal comprising heavy chain immunoglobulin loci where constant and/or variable region
25 elements are replaced with at least a functional portion of the human heavy chain immunoglobulin locus by genetic alteration of a cell nucleus of said animal, introducing said cell nucleus into an enucleated nuclear transfer unit cell to provide a first embryonic stem cell, introducing said first
30 nuclear transfer unit cell into a female recipient host to produce a first mutated neonate;

producing a second mutated animal comprising light chain immunoglobulin loci where constant and/or variable region
elements are replaced with at least a functional portion of
35 the human light chain immunoglobulin locus by genetic alteration of a cell nucleus of said animal, introducing said

cell nucleus into an enucleated nuclear transfer unit cell to provide a second embryonic cell stem cell, introducing said second nuclear transfer unit cell into a female recipient host to produce a second mutated neonate; and

- 5 breeding mature first and second mutated neonates and selecting animals capable of producing substantially human antisera and being at least substantially incapable of producing endogenous antisera.

- 10 27. A method of producing a transgenic nonhuman animal weighing at least 1 kg and comprising human immunoglobulin genes integrated by homologous recombination into its genome, wherein said animal predominantly produces functional, substantially human antibody molecules comprised at least in
15 part of human immunoglobulin polypeptide sequences when immunized, said method comprising:

- producing a mutated animal comprising heavy and light chain immunoglobulin loci where constant and/or variable region elements are replaced with at least a functional
20 portion or the human heavy and/or light chain immunoglobulin locus by genetic alteration of a cell nucleus of said animal, introducing said cell nucleus into an enucleated nuclear transfer unit cell to provide a embryonic cell stem cell, introducing said nuclear transfer unit cell into a female
25 recipient host to produce mutated neonate; and

- breeding mature mutated neonates and selecting animals capable of producing substantially human antisera and at least substantially incapable of producing endogenous antisera.

30

28. The method according to Claim 26 or 27, wherein said nuclear transfer unit cell is an oocyte.

29. The method according to Claim 26 or 27, wherein said
35 animal is from the order of *Lagomorpha*.

30. A method according to Claim 26 or 27, wherein said heavy chain locus comprises at least one constant region element.

31. A method according to Claim 26 or 27, wherein said heavy
5 chain locus comprises at least one variable region element.

32. A method according to Claim 26 or 27, wherein said heavy chain locus comprises the variable region element proximal to the D region.

1/3

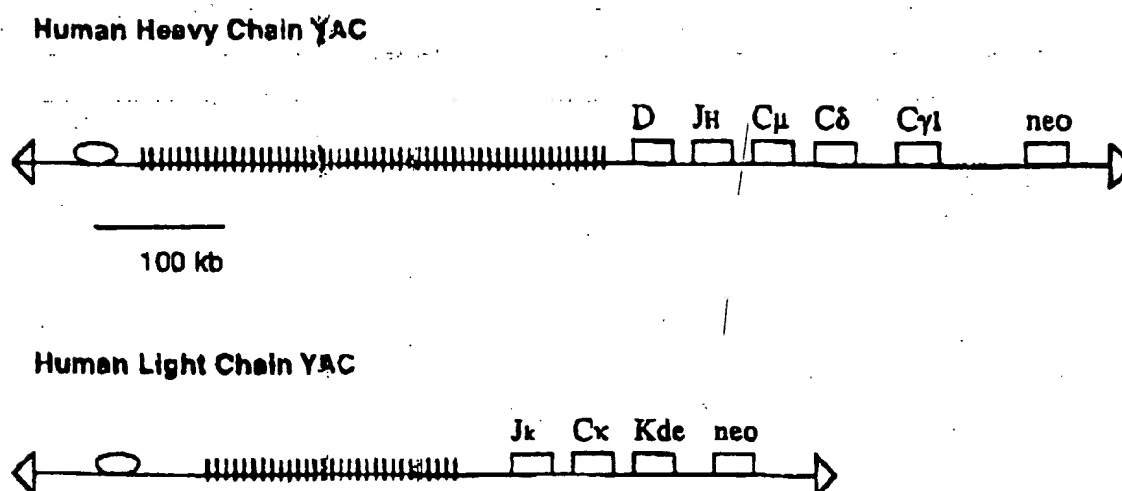


FIG. 1

2/3

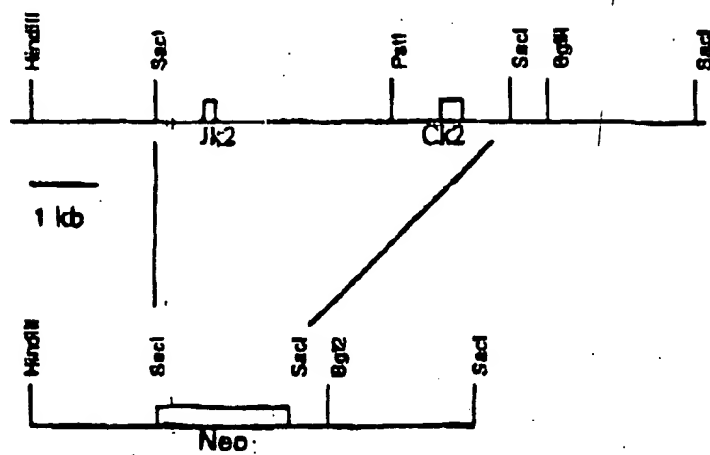
Targeted Inactivation of the K2 gene by JkCk deletion**Targeted rabbit sequence****Targeting construct**

FIG. 2

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 August 2000 (10.08.2000)

PCT

(10) International Publication Number
WO 00/46251 A3

(51) International Patent Classification?: C07K 16/08,
16/18, 16/28, 16/30, 16/24, A61K 39/395, A01K 67/027

(21) International Application Number: PCT/EP00/00933

(22) International Filing Date: 4 February 2000 (04.02.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/118,810 5 February 1999 (05.02.1999) US
60/131,398 28 April 1999 (28.04.1999) US
60/134,674 18 May 1999 (18.05.1999) US

(71) Applicant and

(72) Inventor: BÜLOW, Jens-Ulrich [DE/DE]; Flughafen-
strasse 19, D-76140 Karlsruhe (DE).

(74) Agent: OTTEVANGERS, S.U.; Vereenigde, Nieuwe
Parklaan 97, NL-2587 BN The Hague (NL).

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— With international search report.

(88) Date of publication of the international search report:
11 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HUMAN POLYCLONAL ANTIBODIES FROM TRANSGENIC NONHUMAN ANIMALS

(57) Abstract: Substantially human antisera are provided by genetically modifying a domestic animal generally weighing at least about 1 kg. The domestic animal is genetically modified by generating inactive heavy and light chain immunoglobulin loci and integrating at least functional portions of the human heavy and light chain immunoglobulin loci, whereby the human loci generate an immune response. The antisera find use in the treatment of diseases, immunocompromised patients and in case of transplantation.



WO 00/46251 A3

INTERNATIONAL SEARCH REPORT

In International Application No
PCT/EP 00/00933

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/08 C07K16/18 C07K16/28 C07K16/30 C07K16/24
A61K39/395 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 814 318 A (KAY ROBERT M ET AL) 29 September 1998 (1998-09-29) cited in the application column 1-41 examples 1,4,7,15,22 claim 1 ---	1-32
X	WO 90 04036 A (AGRICULTURAL & FOOD RES ;BRUGGEMANN MARIANNE (GB); MEDICAL RES COU) 19 April 1990 (1990-04-19) page 1-6; examples 1,3 ---	1-32
A	WEIDLE U H ET AL: "Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs." GENE, (1991 FEB 15) 98 (2) 185-91. , XP000919055 the whole document --- -/--	1-32

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *S* document member of the same patent family

Date of the actual completion of the international search

18 October 2000

Date of mailing of the international search report

27. 10. 00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mennessier, T

INTERNATIONAL SEARCH REPORT

In: International Application No
PCT/EP 00/00933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FIRST N L: "New animal breeding techniques and their application." JOURNAL OF REPRODUCTION AND FERTILITY. SUPPLEMENT, (1990) 41 3-14. REF: 103 , XP000941129 the whole document -----</p>	1-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/00933

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 20-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the antisera composition.
2. ☒ Claims Nos.: 1, 5-10 and 20-25 (all partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I:2

Claims Nos.: 1, 5-10 and 20-25 (all partly)

In claim 1 the term "transgenic" is not present and it is referred to a "nonhuman animal" whereas in claims 2-4 which are dependent on said claim the phrase "The polyclonal antisera according to claim 1, wherein said transgenic nonhuman animal" has been used, which means that the nonhuman animal referred to in claim 1 should be a "transgenic" one.

In view of this discrepancy between claim 1 and claims 2-4, and taking into consideration the fact that the description refers to transgenic animals only, the search has been limited with respect to claims 1, 5-10 and 20-25 to those embodiments which are defined with reference to transgenic nonhuman animals.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/00933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5814318 A	29-09-1998	US 5661016 A	26-08-1997
		US 5545806 A	13-08-1996
		US 5789650 A	04-08-1998
		US 5569825 A	29-10-1996
		AU 6819494 A	21-11-1994
		CA 2161351 A	10-11-1994
		EP 0754225 A	22-01-1997
		JP 8509612 T	15-10-1996
		WO 9425585 A	10-11-1994
		US 5625126 A	29-04-1997
		US 5770429 A	23-06-1998
		AU 1318299 A	01-04-1999
		AU 3907895 A	29-02-1996
		AU 3328493 A	19-07-1993
		AU 720612 B	08-06-2000
		AU 4190297 A	15-01-1998
		CA 2124967 A	24-06-1993
		EP 0746609 A	11-12-1996
		JP 7503132 T	06-04-1995
		WO 9312227 A	24-06-1993
		US 5877397 A	02-03-1999
		AT 158021 T	15-09-1997
		AU 8507191 A	30-03-1992
		CA 2089661 A	01-03-1992
		DE 69127627 D	16-10-1997
		DE 69127627 T	19-02-1998
		DK 546073 T	02-02-1998
		EP 0546073 A	16-06-1993
		EP 0814159 A	29-12-1997
		ES 2108048 T	16-12-1997
		GB 2272440 A	18-05-1994
		GR 3024701 T	31-12-1997
		JP 2938569 B	23-08-1999
		JP 6500233 T	13-01-1994
		KR 241638 B	01-02-2000
		WO 9203918 A	19-03-1992
		US 5874299 A	23-02-1999
		US 5633425 A	27-05-1997
WO 9004036 A	19-04-1990	AT 138104 T	15-06-1996
		AU 4417389 A	01-05-1990
		DE 68926508 D	20-06-1996
		DE 68926508 T	31-10-1996
		EP 0438474 A	31-07-1991
		JP 4500911 T	20-02-1992
		KR 164608 B	15-01-1999
		US 5545807 A	13-08-1996